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Molecular and morphologic characterization of *Sarcocystis felis* (Apicomplexa: Sarcocystidae) in South American wild felids from Brazil

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ABSTRACT

Wild felids are thought to share parasites with domestic cats. However, little is known of the coccidian parasites of wild felids. We investigated the presence of *Sarcocystis* spp. in tissues of 6 species of 90 Neotropical small felids killed in road accidents in the state of Rio Grande do Sul, Brazil by using microscopic and molecular techniques. Formalin-fixed tissues from 28 felids were examined, and *Sarcocystis felis*-like sarcocysts were detected in 4 wild cats (2 *Puma yagouaroundi* and 2 *Leopardus guttulus*). By transmission electron microscopy, sarcocysts from a *P. yagouaroundi* were identical to *S. felis* from domestic cats in the USA. Direct sequencing of PCR amplicons resulted the unambiguous sequences of the *ITS-1* region from 18 of the 31 PCR positive wild cats; 5 sequences from each *P. yagouaroundi*, and *Leopardus geoffroyi*, 4 sequences from *L. guttulus*, and 2 sequences from each *Leopardus wiedii*, and *Leopardus colocolo*. Sequences analysis of *ITS-1* region revealed the highest identiy (97–99%) with that of previously describe isolates of *S. felis* from domestic cats in the USA and identified them as *S. felis*. Tissues of 1 *Leopardus pardalis* tested by PCR and histology were negative. The phylogenetic relationship indicated that *S. felis* is quite different to species which employ opossums as their definitive host. This is the first report of *S. felis* infection in small wild felids from Brazil.

1. Introduction

Sarcocystis species have a 2-hosts prey–predator life cycle. Domestic cats are definitive hosts for numerous *Sarcocystis* species of animals, and intermediate hosts for *Sarcocystis neurona* (reviewed in Dubey et al., 2015b). It is generally believed that domestic cats share coccidial species but this has been experimentally proven only for *Toxoplasma gondii* (reviewed in Dubey, 2010). Recently, it was suggested that bobcats (*Lynx rufus*) are a host for *S. neurona* (Verma et al., 2015). *Sarcocystis felis* is another coccidian thought to be shared among wild felids and the domestic cat. Life cycle of *S. felis* is unknown. Dubey et al. (1992) named *S. felis* after finding it in 4 bobcats (*Felis rufus*) from the National Zoo in Washington D.C., USA. Subsequently, *S. felis*-like parasite was thought to be present in lion (Dubey and Bwangamoi, 1994), and cheetah (Briggs et al., 1993); but details are scanty. There are several reports of sarcocysts in domestic cats; all of them except 1 were from the USA (Dubey et al., 2015a). Torres et al. (1996) found

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sarcocysts in diaphragm of 1 of 24 domestic cats in Chile. *S. felis* sarcocysts are up to 2 mm long, the sarcocyst wall is ~1 μm thick, and villar protrusions are short and finger-like with blunt villar trip (Dubey et al., 1992).

There is no report of sarcocysts in muscles of domestic and wild felids from South America. Brazil a natural habitat for several species of wild felids, is not indigenous to North America. The objective of the present study was to characterize molecularly and morphologically sarcocysts found in road killed wild felids.

2. Materials and methods

2.1. Wild felids surveyed

Tissues of 90 Neotropical small wild felids that were killed in road accidents in the state of Rio Grande do Sul, Brazil were used for the present paper (Table 1). The animals had been deposited, and catalogued in five biological collections from within the state between 1999 and 2010. Carcasses were individually wrapped in plastic bags and stored at −20 °C. The following variables were recorded whenever available: origin, date of deposit, species, gender, and age (Palacios, 2007). A total of 345 tissue samples were collected from 90 animals at necropsy (Auricchio et al., 2014); samples were obtained from the quadriceps femoris muscles (83), tongue (56), diaphragm (56), heart (63), ocular muscles (44), and brain (43).

2.2. Microscopic examination

Formalin-fixed tissues from 28 wild cats were shipped to the Animal Parasitic Diseases Laboratory, USDA, Beltsville, Maryland for diagnosis. The fixed tissues were embedded in paraffin, sectioned 5 μm thick and stained with hematoxylin and eosin (H and E). Individual sarcocyst morphology was recorded under light microscope. After examination of the H and E sections, sarcocysts positive tissues embedded in paraffin were excised with the help of a scalpel and processed for transmission electron microscopic (TEM) examination as described by Dubey et al. (2015a).

2.3. Molecular and phylogenetic analyses

Depending on availability, 1 g of tissue was macerated in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) at a 1:4 ratio, and two 200 μL aliquots were used for the molecular detection of parasites. DNA extraction was performed using proteinase K followed by phenol–chloroform (1:1) purification (Pena et al., 2006). The DNA was precipitated with 70% ethanol, resuspended in TE buffer (50 μL, pH 8.0), and stored at −20 °C until use.

The amplification of the partial internal transcribed spacer 1 (*ITS-1*) sequence was performed by polymerase chain reaction (PCR) using JS4 as external forward primer (5′-CGAAATGGGAAGTTTTGTGAAC-3′) and CT2c as external reverse primer (5′-CTGCAATTCACATTGCGTTTCGC-3′), followed by *nested*-PCR (*n*-PCR) using JS4b as forward primer (5′-AGTCGTAACAAGGTTTCCGTAGG-3′) and CT2b as reverse primer (5′-TTGCGCGAGCCAAGACATC-3′). These primers were designed especially for parasite of subfamily Toxoplasmatinae (Slapeta et al., 2002; Soares et al., 2011), which can also detect DNA of subfamily Sarcocystinae. We are reporting first time the use of these primers for amplification of DNA of subfamily Sarcocystinae.

The PCR and *n*-PCR mixtures consisted of a 25 μL reaction volume, including *Taq* DNA polimerase (5 U/μL) (Invitrogen™, California, USA), 10× reaction buffer (50 mM KCl; 10 mM Tris–HCl, pH 9.0), $MgCl_2$ (50 mM), dNTPs (10 mM), forward and reverse primers (10 pM), and 2.5 μL of sample DNA. PCR and *n*-PCR programs consisted of an initial cycle at 94 °C for 3 min, followed by 35 cycles at 94 °C for 40 s, 56 °C for 30 s, and 72 °C for 30 s, with *T. gondii* (RH-1) and *Neospora caninum* (Nc1) as positive controls. The resulting amplicons were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide, and the expected band size for the subfamily Sarcocystinae is ~1000 bp.

The *n*-PCR products were purified with ExoSAP-IT® USB (Affymetrix, Cleveland, USA) following the manufacturer's instructions and sequenced with primers JS4b and CT2b using BigDye® Terminator v3.1Cycle Sequencing Kit (Applied Biosystems®, California, USA), and quantified using an ABI 3500 sequence detection system (Applied Biosystems®, California, USA). The sequences were analyzed and edited using BioEdit software and identified by comparison with sequences available in NCBI GenBank by BLASTn analysis.

Multiple sequence alignment was built by selecting MUSCLE algorithm as implemented in Geneious version 8.0.4. The *ITS1* region of *S. felis* from domestic cats in the USA (AY190081 and AY190082) and new sequences obtained from wild cats from Brazil (KC160197–KC160214) were aligned to detect nucleotide polymorphisms among different isolates. The neighbor-joining algorithm applied to Tamura-Nei genetic distances, as implemented by Geneious version 8.0.4 estimated a phylogenetic tree. The phylogenetic tree was constructed based on *ITS-1* sequences obtained from wild cat's tissues from Brazil and previously published sequences of various *Sarcocystis* species using software Geneious version 8.0.4. Input sequences were the *ITS-1* regions of different *Sarcocystis* species and *T. gondii* retrieved from NCBI GenBank. Tree was tested by selecting bootstrap method with value of 1000 replicates and *T. gondii* (KM657806) as an out group.

2.4. Statistical analysis

The chi-square test was used to determine the association between presence of *Sarcocystis* and host species, gender, age, origin, and date of deposit. The significance level was set at $p < 0.05$ and all analyses were performed using IBM SPSS Statistics19 software.

2.5. Ethics

The study was approved by the Ethics Committee on Animal Use at Federal University of Rio Grande do Sul (UFRGS), Brazil, following the provisions of the Brazilian law on the use of road kill for scientific purposes.

3. Results

Sarcocysts were found in four (2 *Puma yagouaroundi*, 2 *Leopardus guttulus*) of 28 tissues of wild cats by light microscopy. The density of sarcocysts ranged from 1 to 14 per section. All sarcocysts were morphologically similar to *S. felis* sarcocysts (Fig. 1). Longitudinally cut sarcocysts were up to 2 mm long and 48–77 μm wide. The bradyzoites were $8 - 10 \times 2 - 3$ μm ($n = 12$) in size.

The TEM of sarcocyst in *P. yaguaroundi* revealed a 1.5–2.0 μm thick sarcocyst wall with villar protrusions (Fig. 1C, D). The bradyzoites were 11–12 μm long. Irregularly spaced villi were present on the outside of primary wall that displaying short knob-like or dome shaped protusions. The ground substance (gs) of the cyst wall consisted of fine granules without microtubules.

The PCR amplification of *ITS-1* locus was found positive in 31 (34.4%) of the 90 wild cats evaluated. Amplification was detected in 49 (14.2%) of the 345 tissue samples examined (31 of 90 wild felids), mainly in quadriceps femoris muscles (22/83, 26.5%; $p < 0.002$) and tongue (13/56, 23.2%; $p < 0.157$), and to a lesser extent, in ocular muscles (6/44, 13.6%), diaphragm (6/56, 10.7%), brain (1/43, 2.3%), and heart (1/63; 1.6%) (Table 2).

Table 1
PCR amplification of the *ITS-1* locus in tissue samples from 90 Neotropical small cats from Rio Grande do Sul, Brazil.

Variable	Animals examined	Positive animals (%)	Significance level *p*
Species			0.247
Puma yagouaroundi	22	6 (6.7)	
Leopardus geoffroyi	22	8 (8.9)	
Leopardus guttulus	28	7 (7.8)	
Leopardus wiedii	10	6 (6.7)	
Leopardus pardalis	1	0 (0.0)	
Leopardus colocolo	7	4 (4.4)	
Gender			0.836
Male	41	14 (15.5)	
Female	15	4 (4.4)	
Data not available	34	13 (14.4)	
Age			0.682
Adult	52	15 (16.6)	
Juvenile	4	1 (1.1)	
Data not available	34	5 (16.6)	
Origin			0.293
Data not available	26	5 (5.5)	
Mesoregion			
Mideast	2	2 (2.2)	
Midwest	15	4 (4.4)	
Metropolitan	12	6 (6.6)	
Northeast	4	1 (1.1)	
Northwest	6	1 (1.1)	
Southeast	11	6 (6.6)	
Southwest	14	6 (6.6)	
Date of deposit			0.778
1999–2005	22	6 (6.6)	
2006–2010	46	19 (21.1)	
Data not available	22	6 (6.6)	

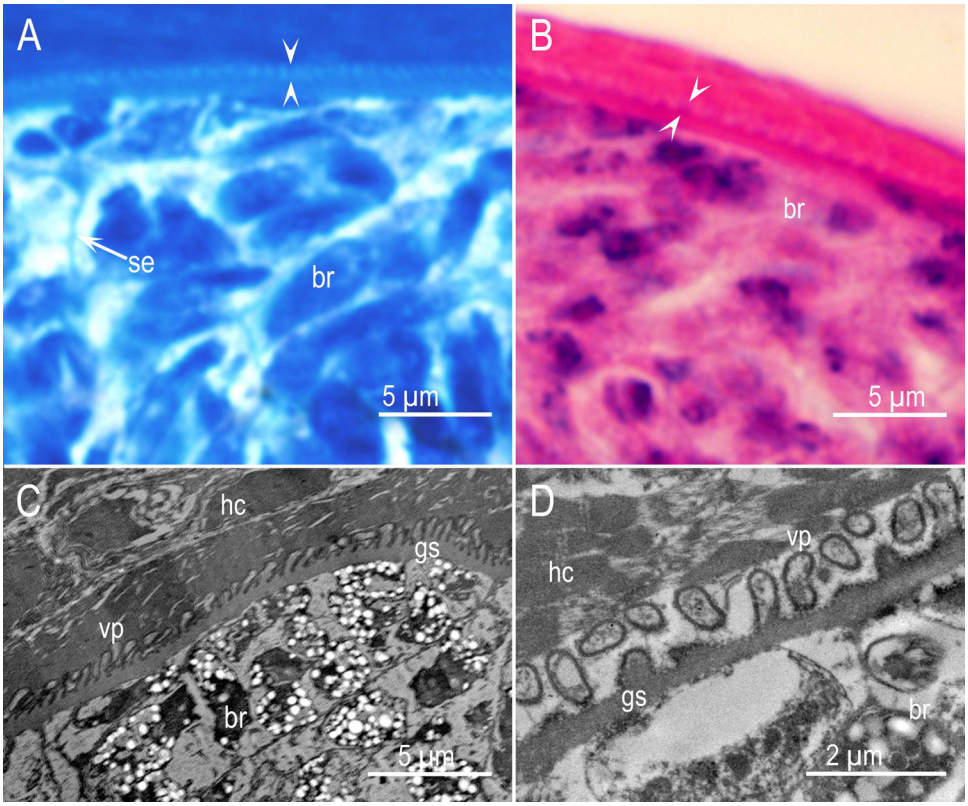

Fig. 1. *Sarcocystis felis* in section of tongue from a *Puma yagouaroundi* from Brazil. (A, B) Light microscopic images of cyst wall in section stained with Toluidine blue (A), and hematoxylin and eosin (B). Note villar protrusions (opposing arrowheads) on the cyst wall, septum (se) and bradyzoites (br). (C, D) Transmission electron microscopy of the cyst wall showing host cell (hc), villar protrusions (vp), ground substance layer (gs), and bradyzoites (br). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Summary of tissue samples from Neotropical small cats from Brazil positive by amplification of the *ITS-1* locus.

Species	Skeletal muscle			Tongue			Diaphragm			Heart			Brain			Ocular muscles			Total		
	E	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%
Puma yagouaroundi	21	4	0.0	15	2	1.3	14	1	7.1	14	0	0.0	11	0	0.0	11	0	0.0	86	7	8.1
Leopardus geoffroyi	21	5	23.8	13	3	23.1	14	2	14.3	14	1	7.1	10	0	0.0	11	3	27.3	83	14	16.9
Leopardus guttulus	26	7	26.9	16	2	12.5	17	1	5.9	21	0	0.0	12	1	8.3	13	1	7.7	105	12	11.4
Leopardus wiedii	8	3	37.5	8	5	62.5	9	2	22.2	10	0	0.0	7	0	0.0	7	1	14.3	49	11	22.5
Leopardus pardalis	1	0	0.0	1	0	0.0	0	0	0.0	0	0	0.0	1	0	0.0	0	0	0.0	3	0	0.0
Leopardus colocolo	6	3	50.0	3	1	33.3	2	0	0.0	4	0	0.0	2	0	0.0	2	1	50.0	19	5	26.3
Total	83	22	26.5	56	13	23.3	56	6	10.7	63	1	1.6	43	1	2.3	44	6	13.6	345	49	14.2

E: number of samples examined, *P*: number of positive samples, %: positivity rate.

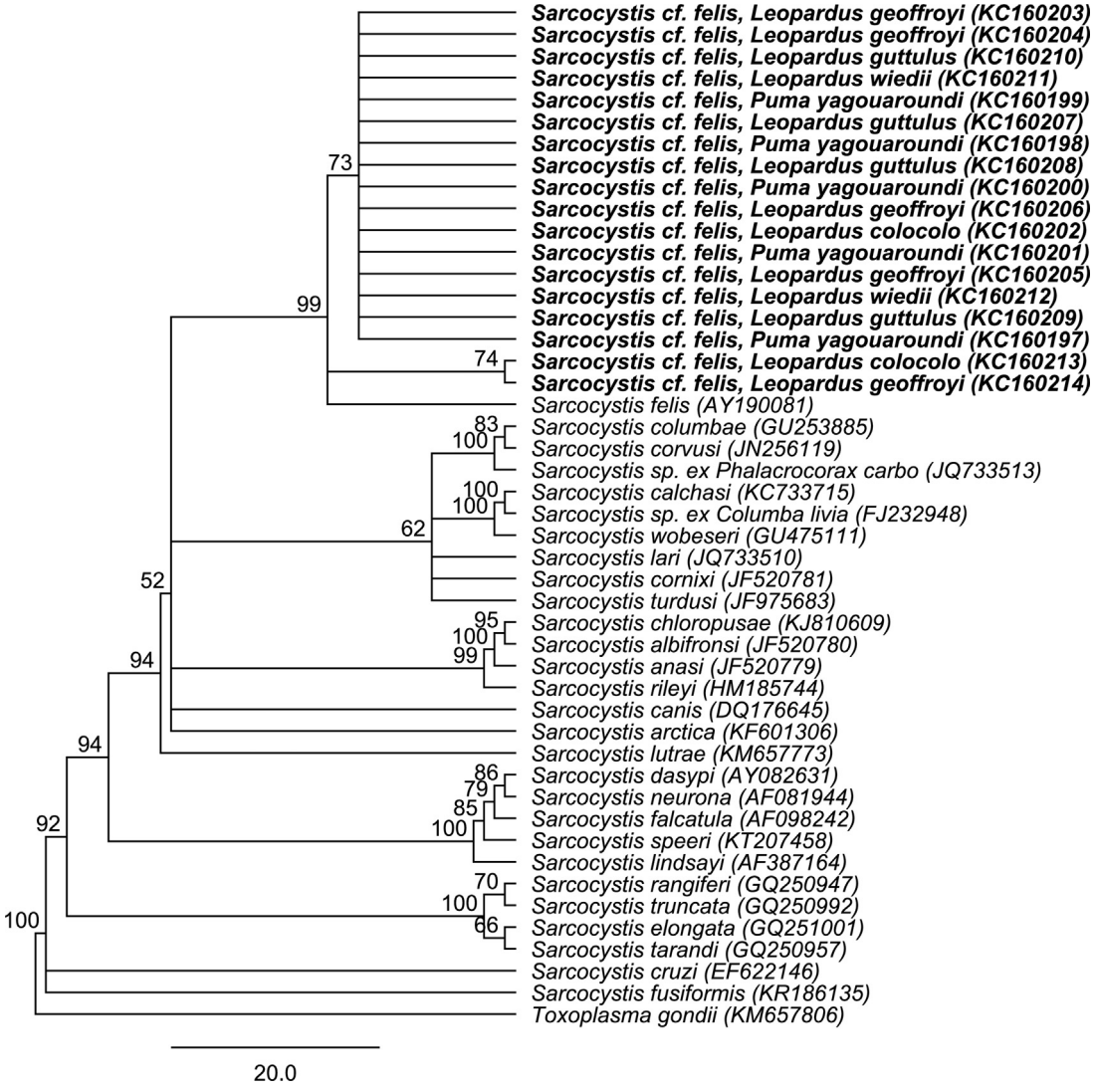

Fig. 2. Phylogenetic tree based on *ITS-1* sequences. Input sequences were the *ITS-1* regions of various species of *Sarcocystis* retrieved from NCBI GenBank, and new *ITS-1* sequences obtained from wild cat's tissues from Brazil. Accession numbers of sequences are given in parenthesis following the species name. Tree was built by selecting the Tamura-Nei genetic distance model and neighbor-joining tree methods (Geneious version 8.0.4). Tree was tested by selecting bootstrap method with value of 1000 replicates and *Toxoplasma gondii* (KM657806) as an out-group. All sequences (KC160197–KC160214) obtained from wild cats tissues from Brazil clustered together in the same branch with previously reported sequence of *S. felis* (AY190081).

The partial *ITS-1* sequences were successfully obtained from 18 different wild cats; 5 sequences from each *P. yagouaroundi*, and *Leopardus geoffroyi*, 4 sequences from *L. guttulus*, and 2 sequences from each *Leopardus wiedii* and *Leopardus colocolo* (Fig. 3). The comparative analysis of sequences showed the highest molecular identity to *S. felis* (GenBank™ sequences AY190081 and AY190082) with 97–99% similarity, corresponding to clones 1453b and 1454 isolated from domestic cats in the United States. The partial *ITS-1* sequences of Brazilian *S. felis* samples examined in this study were deposited in NCBI GenBank™ under accession numbers KC160197–KC160214. The sequences from remaining wild cats were not readable due to mixed chromatograms, and the applied primers were not species specific (Soares et al., 2011). Attempt to obtain unambiguous sequence from remaining wild cats including

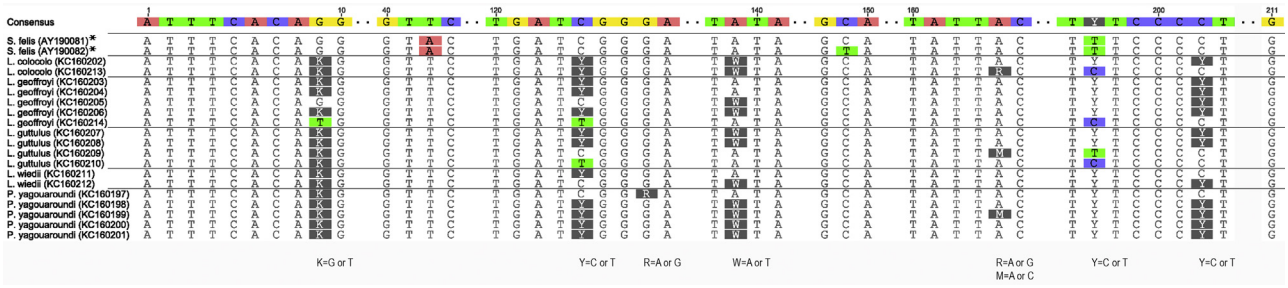

Fig. 3. Multiple sequence alignment of the *ITS-1* region of *S. felis* from domestic cats in the USA (AY190081 and AY190082) and new sequences obtained from wild cats from Brazil (KC160197–KC160214). The alignment was built by selecting MUSCLE algorithm as implemented in Geneious version 8.0.4. The single nucleotide polymorphisms (SNP) at six nucleotide positions among these sequences that uses K (G or T), Y (C or T), R (A or G), W (A or T), and M (A or T); indicating uncertainty or a potential of two different nucleotides at particular site. * used as reference sequences for *S. felis*.

the sample which was examined by TEM was unsuccessful. It may be related to quality and quality of DNA sample; DNA was extracted from host tissues not from cultured parasites or individual sarcocyst.

Multiple sequence alignment of the *ITS-1* region established that *S. felis* from Brazilian wild cats were distinguishable from *S. felis* from domestic cats in the USA by single nucleotide polymorphisms (SNPs) at six nucleotide positions. These nucleotide differences among sequences were substitutions of nucleotides that uses K (G or T), Y (C or T), R (A or G), W (A or T), and M (A or T); indicating uncertainty or a potential of two different nucleotides at particular site (Fig. 3).

Phylogenetical analysis positioned all sequences (KC160197–KC160214) obtained from wild cats tissues from Brazil in the clade with previously reported sequence of *S. felis* (AY190081).

4. Discussion

The sarcocysts in the present study were identified as *S. felis* based on morphologic and molecular characteristics. Morphologically, sarcocysts were identical to *S. felis* sarcocysts described from wild felids and domestic cats from the USA (Dubey et al., 1992). Little is known of clinical significance of *S. felis* in wild felids but immunosuppresion has been associated with reports of sarcocystoisis in domestic cats in the USA, and *S. neurona* can cause encephalitis in cats (Dubey et al., 2015a). Molecular distinction of *S. felis* from *S. neurona* is diagnostically important. Comparative molecular phylogeny between *S. felis* and *S. neurona* from cat sarcocysts was reported previously (Gillis et al., 2003,b; Elsheikha et al., 2006a,b). Over the ssurRNA sequences, *S. felis* and *S. neurona* are closely related to each other (Elsheikha et al., 2006b). However, *S. felis* was found quite different from *S. neurona* and other *Sarcocystis* species at the *ITS-1* region suggesting the species divergence (Gillis et al., 2003; Elsheikha et al., 2006b). The phylogenetic relationship indicated that *S. felis* is not related to species which employ opossums as their definitive host (*S. neurona*, *Sarcocystis dasypi*, *Sarcocystis speeri*, *Sarcocystis falcatula*, and *Sarcocystis lindsayi*) (Fig. 2).

The *ITS-1* region shows much higher level of sequence divergence in comparison to *18S rRNA*, and *28S rRNA* loci, and has been used in several studies to discriminate very closely related *Sarcocystis* species (Dubey et al., 2015a). The applied primers were not species specific (Soares et al., 2011), and there is a necessity to distinguish *S. felis* infections using specific molecular markers. However, PCR-DNA sequences analysis can diagnose infections by *S. felis* versus other especies of *Sarcocystis*. In the present study, the *ITS-1* region was amplified by PCR using DNA extracted from tissues of 31 of 90 wild cats. The partial *ITS-1* sequences were successfully obtained only from 18 different wild cats (211 bp from 14, 565 bp from 1, and 792 bp from 1) (Fig. 3). A total of 5 *ITS-1* sequences was obtained from *P. yagouaroundi* (KC160197- KC160201), and *L. geoffroyi* (KC160203-KC160206, KC160214), 4 sequences from *L. guttulus* (KC160207–KC160210), and 2 sequences from each *L. wiedii* (KC160211, KC160212), and *L. colocolo* (KC160202, KC160213). The sequences from the remaining wild cats were not readable due to mixed chromatograms. Attempts to obtain unambiguous sequence from remaining wild cats including the sample which was examined by TEM were unsuccessful. It may be related to quality and quality of DNA sample; DNA was extracted from host tissues not from cultured parasites or individual sarcocyst. Additionally, the applied primers were common to all parasite of subfamily Toxoplasmatinae; not species specific; amplicons are potentially produced by more than one protozoans co-infecting to individual felids. The analysis of *ITS-1* sequences obtained from wild felids from Brazil diagnose the infection by *S. felis* parasite that show an especially close relationship to *S. felis* from domestic cats in the USA (Figs. 2 and 3).

Based on molecular finding and the microscopic results, the present study provides evidence that these small wild cats in Brazil are natural intermediate host of *S. felis*. The infection is prevalent in the state of Rio Grande do Sul, Brazil and probably other felids populations in South America.

Conflict of interest

The authors declare no conflict of interest.

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